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蘑菇子实体内杀虫蛋白质的研究

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摘要：许多蘑菇都对昆虫表现出毒性。为了证实与杀虫毒性有关的化合物，对14种蘑菇的毒性在水溶性、热敏性和可透析性等方面进行了研究。研究数据表明，蛋白质对大多数蘑菇子实体的杀虫活性起着重要作用，也许是一种可以用于植物抵抗害虫的基因源。在数种蛋白质中，凝集素和溶血素因不受蛋白酶的影响而成为良好的杀虫剂候选材料。

关键词：蘑菇；杀虫剂；蛋白质；凝集素；蛋白酶抑制剂；溶血素
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Proteins as Active Compounds Involved in Insecticidal Activity of Mushroom Fruitbodies

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Abstract：Numerous mushrooms are toxic to insects. To identify the chemicals involved in insecticidal activity，the toxicity of 14 species has been studied for water solubility，thermolability，and dialysis. The data strongly suggest that proteins are responsible for most of the insecticidal avtivity in mushroom fruitbodies and may be a source of genes available for plant protection against insects. Among proteins，lectins and haemolysins are good insecticide candidates because the toxicities are not affected by protease.

Key words：Mushrooms；Insecticide；Protein；Lectin；Serpin；Haemolysin

Mushrooms represent a potential source of chemicals for phytoprotection that can be either organic compounds with insecticidal properties or genes encoding toxic proteins. Several mushrooms such as those belonging to the *Lepista* or *Cantharellus* genera are not eaten by insects，or when a mushroom is attacked by only few species（Bruns，1984）. Therefore，we conducted toxicological screening with the sporophores of higher fungi from southwestern France to find toxic species from which insecticidal compounds could be isolated. We found that numerous species of mushrooms are toxic to two insects assayed *Spodoptera littoralis*（Boisduval）（Lepidoptera：Noctuidae）and *Drosophila melanogaster*（Meigen）（Diptera：Drosophilidae）when powdered fruitbodies were incorporated into rearing medium

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(Mier *et al*, 1996). These two insect species were choosen as models of two orders that contained numerous economic pests.

Some insecticidal compounds have already been isolated from mushrooms. Amatoxins and phallotoxins from *Amanita phalloides* (Fr.) Link, are toxic to many insects (Jaenike *et al*, 1983, Ying *et al*, 1987); ibotenic acid is a fly-killing compound found in *A. muscaria* (L.: Fr.) Pers, *A. pantherina* (DC: Fr.) Krombh,; L – DOPA has been isolated from *Strobilomyces strobilaceus* (Scop.: Fr.) Berk and *Hygrocybe conica* (Scop.: Fr.) Kummer (Steglich and Esser, 1973, Steglich and Preus, 1974); and the nucleoside clitocine has been isolated from *Lepista inversa* (Scop.) Patouillard (Kubo *et al*, 1986). But, until now, among these mushrooms studies no any insecticidal properties had been assigned to proteins, although several proteins with potential defensive action against insects have been isolated from plants, for example, lectins (Janzen *et al*, 1976), protease inhibitors (Ryan, 1973, 1978, 1989; Pilgrim *et al*, 1992), and lipoxygenases (Shukle and Murdock, 1983). In this study, we report evidence that most of the insecticidal properties of mushrooms are due to proteins.

Materials and Methods

Preparation of Crude Extract Fruitbodies of fourteen mushroom species were collected in southwestern France and kept frozen at – 20℃ until used. They were crushed in distilled water (1: 1.5, wt: vol) and centrifuged for 20 min at 6 500 × *g* to remove undissolved materials. Protein concentrations of the extracts were estimated with Bradford reagent and bovine serum albumin as reference.

Toxicological Test with *Drosophila melanogaster* Larvae We used *D. melanogaster* larvae as toxicological test organisms because they are effective model to test insecticidal compounds although this insect does not represent an economic problem. *Drosophila melanogaster* has a short life span and is easy to rear. Determination of toxicity was performed after ingestion. Various amounts of each extract were added to 1 mL of rearing medium (10% yeast [Prolabo, France], 2% agar [Colloïdes Naturels International, France], 0.5% para-methyl-hydroxy-benzoic acid [Research Organics Inc., Cleveland, Ohio]) before pouring into 5 mL tubes. Ten eggs of Canton S strain were deposited on the medium and the tubes were maintained at 25℃ for 2 wk to allow larvae development. Numbers of pupae were recorded in the tubes containing at least five concentrations of extract resulting in between 0 and 100% mortality.

Each toxicological test was replicated between three and ten fold. In the reference assay, Without mushroom extract, 90% of the eggs developed through to the pupa stage. Corrected mortality due to the added extract was estimated with Abbott's formula: mortality = (mortality in the sample-mor fitting the log of concentration versus mortality data to sigmoid curves blinear regression and are expressed in microliters of crude extract.

Lectin and Haemolysin Activity Human erythrocytes (group A) were washed three times in phosphate buffer saline (PBS) (0.15 mol NaCl, 10 mmol [Na_2HPO_4, H_2NaPO_4] pH 7.2) and collected by centrifugation (3 500 r/min, 20 min). The pellet was used to prepare a suspension of 4% (vol: vol) erythrocytes in PBS. Determination of the agglutination activity, i. e. lectin activty, was carried out in a final volume of 225 μL. The sample was serially diluted in PBS, with 2-fold increments to obtain a volume of 200 μL; 25 μL of the erythrocyte suspension was then added. The tubes were left for 2 h at room temperature and the agglutination monitored visually. Haemolysis activity corresponds to the minimum amount of extract needed to lyse the erythocytes and agglutination activity corresponds to the minimum amount of extract needed to precipitate the erythrocytes.

Table 1 Water solubility of the active compounds

species	LD_{50} of lst supernatant (μL)	LD_{50} of lst pellet (μL)	LD_{50} of 3rd pellet (μL)	Toxicity extracted with water (%)
Amanita phalloides	2	57	1430	99.9
Xerocomus chrysenteron	3.6	62.5	59	94
X. subtomentosus	14	78	106	89
Lepista nuda	21	81	357	95
Xerocomus badius	43	75	270	90
Boletus aemilli	45	110	345	90
Hygrophoropsis aurantiaca	73	120	2000	98
Clitocybe nebularis	77	1250	3300	98
Polyporus Squamosus	100	714	714	88
Boletus aereus	103	500	555	85
Clitopilus prunulus	105	227	555	87
Albatrellus cristatus	131	454	588	83
Serpula lacrymans	154	625	2000	94
Hygrophorus chrysodon	312	3333	10000	97

Table 2 Proteins and activity extracted from each mushroom

Species	protein (mg/mL)	Haemolysin (μL)	Lectins (μL)	Serpins (μL)
Amanita phalloïdes	1.6	1.56	> 1.56	–
Xerocomus chrysenteron	5.3	6.25	0.78	83
X. subtomentosus	10	–	1.56	–
Lepista nuda	3.8	50	3.1	–
Xerocomus badius	8.6	12.5	3.1	–
Boletus aemilli	1.4	25	1.56	–
Hygrophoropsis aurantiaca	3.9	50	> 50	–
Clitocybe nebularis	4.8	50	3.125	330
Polyporus squamosus	5	3.1	> 320	–
Boletus aereus	4.2	50	0.78	330
Clitopilus prunulus	5.3	25	> 25	330
Albatrellus. cristatus	3.3	25	6.25	–
Serpila lacrymans	2.3	100	> 100	–
Hygrophorus chrysodon	1.2	25	0.78	–

Serpin Activity Crude extracts were incubated with 0.1 mg/mL trypsin in 25 mmol Tris – HCl (pH 7.6), 100 mmol NaCl, and 25 mmol $CaCl_2$ at 25℃ for 30 min. Remaining trypsin activities were measured spectrophoto-metrically at 405 nm with 25 μmol Nα-benzoyl-DL-arginine-para-nitroanilide (Sigma-Aldrich Co., St. Louis, MO) in 25 mmol Tris-HCl (pH 7.6), 100 mmol NaCl, and 25 mmol $CaCl_2$ (Ellant *et al*, 1985). Because inhibition followed pseudo first-order kinetics, the IC_{50} (concentration causing 50% inhibition) was determined by fitting the log of the amount of extract against the remaining activity in linear regression.

Heat Treatment and Proteolysis Test Crude extracts were treated at 100℃ in a water bath for 60 min. When precipitation occured, the samples were stirred before toxicological testing. Crude extracts were treated by pronase (protease type XIV: Bacterial, from *Streptomyces griseus*; Sigma-Aldrich Co., St. Louis, MO) at a final concentration of 4 mg/mL in PBS at 37℃ for 2 h. This protease in the rearing medium was not toxic to *D. melanogaster*. For clarity, effect of each treatment was expressed as percentage of remaining activity, for example, percentage toxicity was (LD_{50} after treatment : LD_{50} initial) × 100.

Result and Discussion

Water Solubility Mushrooms of fourteen species were homogenized in water (1:1.5, wt:vol). After centrifugation, pellets were resuspended in the volume of water corresponding to the supernatant. Most of the insecticidal compounds were extracted by water because the LD_{50} was always lower in the

supernatant than in the resuspended pellet (Table 1). However, some toxicity remained in the pellet. Therefore, it was successively crushed in the same volume of water three times. The LD_{50} in the pellet usually increased by additional extractions, suggesting that remaining activity in the pellet was due to water-soluble molecules. There was no exception in the fourteen mushroom species analyzed, and > 85% of the insecticidal activity was extracted by water. We recorded activities of some proteins known to have potential insecticidal properties (Table 2). Haemolysins as lectins were present in most mushrooms. However lectins were only detected when their activities were below the haemolysin activities because aggregation of erythrocytes could be recorded only if cells were not lysed. Serpins activities were only detected in four mushroom species.

Effect of Dialysis on Insecticidal Toxicity To estimate the molecular mass of insecticidal molecules, aqueous extracts were dialyzed against the same volume of 10 mmol phosphate buffer (pH 7) for one night at 4℃, with a 10-kDa cutoff membrane. The interior and exterior contents of the dialysis tubing were compared for their toxicities. If toxicity is due to macromolecules, all toxicity should be retained inside the dialysis tubing. However, if the toxicity is due to small molecules, the toxicity should be equilibrated between two compartments. The majority (> 80% except for *A. phalloides*) of the toxic compounds remained inside the dialysis tubing for all species tested (Table 3). Suggesting that they have molecular masses > 10 kDa. For *Xerocomus chrysenteron* (Bull.) Quélet and *Lepista nuda* (Bull.: Fr.) Cooke, all the toxicity is due to macromolecules. For some others however, especially *A. phalloides*, some toxicity was recovered outside the dialysis tubing in accordance with the presence of toxic cyclic peptides of molecular masses < 10 kDa. However, toxicity was not equally distributed between two compartments. Suggesting that in this species, macromolecules also may be involved in insecticidal activity. As expected, > 85% of proteins (data not shown), recorded by a global staining method, remained inside the dialysis tubing for all mushroom species, as well as, lectin, haemolysin and serpin activities. These results suggest that most insecticidal activities are due to proteins.

Effect of Heat Treatment on Insecticidal Toxicity To confirm protein involvement in insecticidal toxicity, we tested residual activities after heat treatment because heating denaturation is an important characteristic of proteins. As shown in Table 3, after incubation at 100℃ for 60 min, all extracts were completely inactivated. Only 57% of toxic activity was not destroyed by heating in *A. phalloides*. That was the only exception that is in accordance with the presence of thermoresistant peptides such as amanitin and phalloidin. However, 43% of toxic activity was thermolabile. So, even in this species, part of toxicity against insects is due to a heat-labile protein (s) in accordance with dialysis results.

Lectin and haemolysin activities were supressed by the treatment except for *X. badius* (Fr.: Fr.) Gilbert. Identically, serpin activity were supressed in two of four species, *Boletus aereus* Bull.: Fr. and *Clitopilus prunulus* (Scop.: Fr.) Quélet. Thus, heat treatment inactivated most proteins that might be toxic for insects. For *X. chrysenteron* and *Clitocybe nebularis* (Batsch: Fr.) Kummer, heat treatment had differential effect on toxic and serpin activity suggesting that serpins are not involved in

the toxicity.

Effect of Protease on Insecticidal Toxicity　Protease treatment was initially tested with highly purified protease as trypsin or proteinase K. Nervertheless, some protease inhibitors occur in mushrooms (Otto and Lipperheide, 1986; Pilgrim *et al*, 1992). To overcome a potential inhibition of a specific enzyme, we used a mixture of several proteases, the pronase. The efficiency of the protease treatment was recorded by loading crude extracts treated by pronase onto gel electrophoresis under denaturing conditions along with untreated extracts. Results shows that the majority of proteins were digested by proteases, but some remained unaffected. For most species, the toxicities were not modified by protease treatment (Table 3). The same results were obtained with trypsin or proteinase K (data not shown). In some species, toxicitiy was only diminished slightly, such as in *C. nebularis*. In others, toxicity was augmented 2 fold as for *X. subtomentosus* (L.: Fr.) Quélet. Resistance to proteases may be explained by two hypotheses: 1) toxic proteins were degraded to subunits that are toxic or even toxicer than the initial material, 2) toxic proteins were not attacked by proteases or are protected. To test the first hypothesis, crude extracts were treated by proteases and then dialysed against 10 mmol PBS (pH 7) (1∶1; vol∶vol) for one night at 4℃. Toxicity inside and outside the dialysis tubing was estimated. Table 4 shows that the toxic compounds still remained inside the dialysis tubing, indicating that they were not broken down into subunits < 10 kDa by protease. Thus, this result sustained the second hypothesis which is supported by haemolysins and lectins comportment. Their molecular masses were > 10 kDa, and most of them were heat sensitive and unaffected by protease treatment (Table 3). Lectins appeared to be systematically resistant to proteases in crude ex-

Table 3　Effect of crude extract treatments on toxicity and activities of some selected proteins

Species	Toxicity			Lectins			Haemolysins			Serpins		
	Dialysis[a]	100℃[b]	Pronase[b]	Dialysis[a]	100℃[b]	Pronase[b]	Dialysis[a]	100℃[b]	Pronase[b]	Dialysis[a]	100℃[b]	Pronase[b]
Amanita phalloides	63	57	89	–	–	–	100	0	50	–	–	–
Xerocomus chrysenteron	98	3	96	99	0	100	100	0	100	100	83	100
X. subtomentosus	99	< 1	197	100	1	100	–	–	–	–	–	–
Lepista nuda	99	3	65	–	–	–	100	0	25	–	–	–
Xerocomus badius	88	17	61	100	12	100	100	25	50	–	–	–
Boletus aemilli	91	< 3	68	100	50	100	77	50	100	–	–	–
Hygrophoropsis aurantiaca	91	< 17	113	–	–	–	80	0	100	–	–	–
Clitocybe nebularis	82	< 15	46	91	2	100	100	0	100	100	83	0
Polyporus squamosus	92	11	110	–	–	–	100	0	50	–	–	–
Boletus aereus	88	< 10	165	100	0	100	100	25	100	100	0	0
Clitopilus prunulus	89	< 10	105	–	–	–	90	25	100	100	0	0
Albatrellus cristatus	83	< 29	72	100	0	100	77	0	100	–	–	–
Serpila lacrymans	84	< 20	94	100	0	50	100	0	100	–	–	–
Hygrophorus chrysodon	87	< 30	94	100	0	50	100	0	100	–	–	–

Table 4　Effect of dialysis on samples previously treated by pronase

Species	Toxicity, %[a]
Amanita phalloides	49
Xerocomus chrysenteron	98
X. subtomentosus	98
Lepista nuda	80
Xerocomus badius	78
Boletus aemilli	85
Hygrophoropsis aurantiaca	77
Clitocybe nebularis	83
Polyporus squamosus	86
Boletus aereus	88
Clitopilus prunulus	89
Albatrellus. *cristatus*	90
Serpila lacrymans	65
Hygrophorus chrysodon	81

[a]Remaining activity after dialysis expressed as percentage of total activity inside dialysis tubing.

tract. We assume that the protection originated from proteins that are aggregated with the lectins in crude extract because purified lectins were sensitive to proteolysis (data not shown). Thus, proteins are the active compounds responsible for the inseticidal activity of mushroom fruitbodies, with lectins and haemolysins probably playing a major role.

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